Dear Editor,

Hepatitis C virus (HCV) is a positive-strand RNA virus that belongs to the genus *Hepacivirus* within the *Flaviviridae* family. HCV causes chronic liver diseases, and 185 million people are infected (Messina et al., 2015). Currently, there is no approved vaccine to prevent hepatitis C. HCV induces autophagy through elevating reactive oxygen species (ROS) levels via the unfolded protein response (UPR) or via direct interference with the autophagic pathway, which is crucial for the initiation of the HCV infection process (Drexel et al., 2009; Estrabaud et al., 2011; Huang et al., 2013). Autophagy is a highly conserved intracellular process that targets cytosolic components for lysosomal degradation and is initiated by the formation of double-membrane vesicles called autophagosomes (Sica et al., 2015). The autophagosome ultimately fuses with a lysosome to form an autolysosome (Feng et al., 2014).

The retromer complex is a key part of the endosomal protein sorting machinery that transports cargo proteins to their appropriate destination (Burd and Cullen, 2014). Retromer contains two subcomplexes: a cargo-sorting subcomplex and a membrane-deforming subcomplex. The cargo-sorting subcomplex is a trimer of Vps26, Vps29 and Vps35, whereas an SNX heterodimer forms the membrane-deforming subcomplex (Seaman, 2012). Recently, we discovered that the retromer component Vps35 associates with the HCV NS5A protein and that retromer is required for HCV replication (Yin et al., 2012). In the present study, we found that retromer localized to autophagosomes during HCV replication. Thus, we suggested a link between retromer and autophagy in the context of HCV infection.

To perform the experiments, Huh 7.5.1 cells were grown in Dulbecco’s Modified Eagle’s Medium supplemented with 10% fetal bovine serum. Infectious JFH1 plasmid pJFH1 was obtained from Dr. Takaji Wakita. The OR6 cell line, which harbors full-length genotype 1b HCV RNA and co-expresses *Renilla* luciferase and from Drs. Nobuyuki Kato and Masanori Ikeda, was grown in DMEM supplemented with 10% FBS and 500 μg/mL of G418 (Promega, Madison, WI, USA). OR6 cells were cleared of HCV infection by incubating with IFN α (10 IU/mL) for 7 days to generate cured OR6 cells. Chloroquine (CQ) was from Sigma-Aldrich (St. Louis, MO, USA).

To study the location of retromer and autophagosome membrane, we perform the immunofluorescence microscopy assay and live cell imaging assay. In the immunofluorescence microscopy assay, Cells seeded onto glass coverslips were washed with phosphate-buffered saline (PBS) and fixed with 4% formaldehyde in PBS buffer for 5 min at room temperature. Fixed cells were stained as previously described (Li et al., 2014). The antibody against LC3B used for immunofluorescence and immunoblot was from Cell Signaling Technology (Danvers, MA, USA) (catalogue No. 3868).

To quantify the degree of colocalization, Pearson correlation coefficient values were calculated using the WCIF Image J software with the colocalization threshold plug-in. Pearson correlation coefficient data was from 32 OR6 cells or 27 JFH1-infected Huh 7.5.1 cells. For Live cell imaging assay, Huh7.5.1 cells grown to 40 to 50% confluency on a chambered coverglass were transfected with GFP-LC3 and mCherry-SNX1 for 24 hours. Time-lapse fluorescence images were acquired with the Ultraview Live Cell Imaging System (PerkinElmer, Waltham, MA, USA). The GFP-LC3 was provided by Dr. Zhiping Xie (Nankai University, Tianjin, China). The mCherry-SNX1 was cloned to mCherry-C1.

Previous work has suggested that HCV uses the autophagosome membrane for RNA replication (Sir et al., 2012). To investigate the role of retromer in HCV induced autophagy, we examined LC3B, Vps35 and NS3 in the context of HCV infection. LC3B localized to membrane only if the autophagy is started. There is no LC3B signal without HCV in cured OR6 cells (Figure 1A). As shown in Figure 1B and 1C, retromer co-localized with autophagosomal puncta in OR6 (Pearson correlation coefficient of LC3B/Vps35: 0.5624 ± 0.0989, n = 32) or JFH1-infected Huh7.5.1 cells (Pearson correlation coefficient: 0.8450 ± 0.0663, n = 27). To determine whether retromer associated with autophagosomes, we performed live cell imaging experiments to assess the localization of GFP-LC3 (an autophagosome marker) and mCherry-SNX1 (a component of retromer) in Huh7.5.1 cells. As shown in Figure 1D, LC3 and SNX1 could localize to the same vesicles. Taken together, these results suggested an association between retromer and autophagosome.
Retromer in HCV-induced autophagosomes

A

Cured OR6

B

OR6

C

JFH1 Huh7.5.1

D

GFP-LC3 and mCherry -SNX1 Huh7.5.1

E

F

Mock Cured OR6 OR6 OR6

CQ Cured OR6 OR6 OR6

G

Mock Cured OR6 OR6 OR6

CQ Cured OR6 OR6 OR6

Vps35 siRNA Vps26A siRNA Control siRNA

Vps35 Vps26A Vps35 Vps35 Vps35

LC3B-I LC3B-II p62 Vps35 Actin Core

LC3B-I LC3B-II Vps35 Actin

LC3B-I LC3B-II Vps26A Actin
reduced, and HCV replication is consequently inhibited.

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by viral-induced autophagy. As a result, autophagocy-
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romer and autophagy, we measured autophagic flux by

To further understand the relationship between ret-
romer and autophagy, we measured autophagic flux by
inferring the LC3B-II turnover in the presence and ab-
sence of the lysosomal inhibitor chloroquine (CQ), which
inhibits autophagy after autophagosomes have formed
(Feng et al., 2014). As shown in Figure 1F and 1G, in
cured OR6, silencing Vps35 or Vps26A upregulated the
level of LC3B-II compared with control siRNA treat-
ment, indicating increased host autophagy. However, in
the presence of CQ, the amount of LC3B-II in retromer
knockdown or control knockdown OR6 cells is maintained
at similar levels, suggesting that the levels of total autoph-
agy including viral induced autophagy and host autoph-
agy remained the same.

Autophagy is commonly exploited as a viral infection
strategy (Moser von Filseck et al., 2015). HCV can in-
duce autophagic responses and utilize autophagosomal
membranes as sites for the replication of its RNA (Sir et
al., 2012). Retromer associates with autophagosomes
(Dengjel et al., 2012; Popovic et al., 2012). Interestingly,
we noted that retromer colocalized with LC3B in the con-
text of HCV infection, suggesting that retromer may reg-
ulate HCV replication through autophagy. We hypothe-
size that retromer promotes HCV replication by provid-
ing autophagosomal membranes. In HCV-infected cells,
autophagosomal membranes can be divided into two
pools, including host autophagy and viral-induced au-
phagy. Increased host autophagy by silencing retromer
may consume the common membrane resources shared
by viral-induced autophagy. As a result, autophago-
somal membranes, which support HCV replication, are
reduced, and HCV replication is consequently inhibited.

In summary, we propose a model for the role of retromer
in HCV replication. Upon HCV infection, retromer may
provide double-membrane autophagosomal membranes
for HCV replication. Our studies suggested a novel link
between retromer and autophagy in HCV replication,
which may provide new therapeutic targets for antiviral
therapy.

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REFERENCES
